A Prospective, interventional, randomized, double blind, parallel, placebo-controlled clinical study to evaluate the efficacy and safety of *Bacillus clausii* 088AE in the treatment of antibiotic-associated diarrhoea

Research protocol

WHO recommended research protocol layout

PART 1

Project summary

The present randomized controlled clinical trial shall undertaken to evaluate the efficacy and safety of a probiotic *Bacillus clausii* 088AE in improving antibiotic-associated diarrhea (AAD) and related symptoms. The trial shall be conducted on pediatrics (PE, n=60, 2-10 years), adolescent and adults (AA, n=60, 11-65 years) patients suffering from antibiotic associated diarrhea (AAD). Patients shall be administered with *B. clausii* 088AE for a period of seven days (PE, 4 and AA, 6 billion/day, respectively) and primary and secondary endpoints shall be evaluated following study schema.

Rationale	Diarrhoea is a common adverse effect of antibiotic treatment. It is mainly due to the disruption of gastrointestinal microbial ecology. Probiotics are known to restore the microbial balance and clinical improvement in symptoms associated with antibiotic-associated diarrhea.
Objectives	To assess the efficacy and safety of *Bacillus clausii* 088AE in the treatment of antibiotic-associated diarrhea with related gastrointestinal disorders.
Methods	Allocation: Randomized; Intervention Model: Parallel Design; Masking: Double Blind; Primary Purpose: Treatment, Safety; Arm: Double Arm
Populations	Total of 120 patients [paediatric, 60 (2-10 years) and adolescent & adult, 60 (11-65 years)]
Time frame	17±3 days (for treatment)
Expected outcomes	Primary endpoints: • Time to Last Unformed Stool (TTLUS) – At 24 hr, 48 hr, 96 hr, 168 hr. • Number of unformed stools at time points - At 24 hr, 48 hr, 96 hr, 168 hr – starting from first IP administration. • Time to complete resolution of functional gastrointestinal discomforts- At 24 hr, 48 hr, 96 hr, 168 hr • % responders as defined by the number of subjects with complete remission of diarrhoea within At 24 hr, 48 hr, 96 hr, 168 hr

	Secondary Endpoints • To evaluate the changes of the severity of AAD related symptoms [Time Frame: Baseline/Day 1 to EOT] • To evaluate the changes in VAS score of AAD- At 24 hr, 48 hr, 96 hr, 168 hr • Tolerance [Time Frame: Baseline/Day 1 to EOT] • Assessment of safety of the Investigational products I. Adverse event II. Physical examination III. Biomarker of systemic safety - Hematological test (Complete & differential blood count) - Liver function test (SGOT, SGPT) - Renal function test [Serum creatinine, Serum albumin, Serum sodium, Serum potassium, Blood Urea Nitrogen(BUN)] - Biochemical test (RBS, Total cholesterol)

General information

Protocol title, protocol identifying number (if any), and date.

A Prospective, interventional, randomized, double blind, parallel, placebo-controlled clinical study to evaluate the efficacy and safety of *Bacillus clausii* 088AE in the treatment of antibiotic-associated diarrhoea

Name and address of the sponsor/funder.

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Rationale & background information

Treatment with common and new antibiotics is becoming progressively ineffective and complicated due to rapid emergence of antimicrobial resistance. Antibiotic treatment has most prevalent adverse effects such as antibiotic-associated diarrhea and other gastrointestinal, physiological and psychological ailments (1,2). Commonly associated symptoms of antibiotic-associated diarrhea are dehydration, fever, nausea, vomiting, abdominal cramps, watery diarrhea, bloody and foul-smelling diarrhea range from mild and self-limiting to severe. A clinically significant antibiotic-associated diarrhea has minimum three episodes of mushy or watery stools per day right after few hours up to two months of antibiotics intake (3).

Use of antibiotics systematically induct multimodal disturbances of microbial ecology through longitudinal decrease in distribution, diversity and richness of indigenous microbes in gastrointestinal tract (4,5). Aminoglycoside streptomycin perturbs the microbiota balance and increase the abundance of phyla *Bacteroidetes* and *Firmicutes* (6). Ampicillin and ciprofloxacin augments genus *Acinetobacter* and *Lactobacillus*, respectively (7). Certain antibiotics induce the abundance of *Fusobacteria*, *Clostridia* and *Eubacteria* while demolishing beneficial groups like *Bacteroides* and *Bifidobacteria* (8). Such imbalance (*dysbiosis*) affects microbial synergisms by depriving microbial network, nutrients, metabolites of microbial and host origin, promoting horizontal transfer of antibiotic resistant genes (ARGs) and mobile genetic elements (MGEs) to sensitive microorganisms. A collapsed resident microbiota often fails to resist the overgrowth of opportunistic microorganisms that present endogenously and to suppress the pathogenic invasion. Re-establishment of normal gut microbiota happens over time after cessation of antibiotic therapy (9). A recovered microbiota after antibiotic treatment can be unstable and consequently, patients can be susceptible to secondary infections and other diseases. As a result, antibiotic-associated diarrhea may cause prolonged hospitalization with persistent illness and can increase the cost of health care (10).

Restoration of normal gut microbiota is therefore a fundamental therapeutic paradigm where probiotics could offer promising healthcare solution for antibiotic-associated diarrhea (11). Probiotics are potential live bio-therapeutics, which maintain intestinal microecology during or after antibiotic treatment through competition for epithelial receptors and nutrients, inhibition of epithelial and mucosal adhesion of pathogens, producing short chain fatty acids (SCFAs) and balancing non-favorable intestinal pH (low), by modulating immunity and synthesizing antimicrobial peptides and metabolites. With these mechanisms, probiotics offer promising route for treating many gastrointestinal ailments including antibiotic-associated diarrhea without a risk of spreading antibiotic resistance in other microorganisms (12,13).

Therapeutic effects of probiotics in antibiotic-associated and infectious diarrhea have been evaluated through randomized controlled trials (RCTs) and meta-analysis (14). Probiotics interventions are either with monoculture or mixed culture formulations and consisted a variety of microbial species, like, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum, Lactobacillus acidophilus, Bacillus coagulans, Bacillus clausii* and *Saccharomyces boulardii* (15). Among all, probiotic *Bacillus clausii* has been studied for a variety of acute and infectious diarrheal conditions like acute and chronic diarrhea, acute community-acquired diarrhea (ACAD), antibiotic associated diarrhea (AAD), *Clostridium difficile*-induced diarrhea (CDID) across different geographical locations. The adjunctive treatment with *B. clausii* strains has been reported safe among children, adult and elderly population (16). It is currently available as over the counter (OTC) medical product and used as option (17).

B. clausii (average genome size 4197324 - 4598557 bp and 42.8 – 44.75% GC content), a sporogenous probiotic bacteria belongs to genus *Alkalihalobacillus* and widely studied for production of high-alkaline proteases, antimicrobials like type-A lantibiotic clausin (Mw 2107.94 - 5,158.11 Da) and other lantibiotics (18,19). *B. clausii* maintains gut health by improving digestive microenvironment, reprogramming intestinal microbiota and modulating

host's immunity (20-22). The naturally encapsulated coating of bacterial spores gives protection against various drought conditions like high temperature, desiccation, osmolarity, etc. This highly resilient allochthonous probiotic can survive and proliferate in harsh gastrointestinal conditions comprising gastric acid, pepsin, pancreatin, ions, digestive enzymes, bile and mucins (23). The efficacy of probiotic *B. clausii* varies among strains; differs as per dosages used in the finished formulations and severity of clinical conditions in order to exhibit the intended health benefits (12,20,24).

B. clausii strains have long been safely consumed by the general human population (24). *B. clausii* is considered as the Qualified Presumption of Safety (QPS) listed bacteria by European Food Safety Authority (EFSA) (25). *B. clausii* strains show resistance to one or multiple antibiotics; however, the relevant genes are intrinsically present in chromosomal DNA rendering them highly stable, nontransferable and non-functional (26,27). Besides, the absence of extrachromosomal genome makes *B. clausii* a risk-free and safe probiotic for human and animal applications28. The intrinsic antibiotic resistance in probiotics can be advantageous as these probiotics can be used in combination with antibiotics to restore microbiota in various gastrointestinal ailments including antibiotic-associated diarrhea (24,29).

Comparative evaluation on *B. clausii* strains depicts a heterogeneous and variable clinical benefit in different diarrheal conditions. Such effects happen primarily due to differential ability of strains to survive and germinate in harsh gastrointestinal conditions, adhesion and colonization on epithelial cells, to modulate immune system, varying compatibility to complex treatment conditions and host adaptability (30). *B. clausii* strain with fulfilment of these attributes may bestow a consistent therapeutic outcome in gastrointestinal illnesses henceforth this study is originated. The current study shall evaluate the clinical efficacy and safety of the probiotic *B. clausii* 088AE [MCC 0538] in improving antibiotic-associated diarrhea and related symptoms in pediatrics and adolescent and adult population. *B. clausii* 088AE (genome size 4598557 bp, GC content 44.74%, NCBI Reference Sequence No. CP031128 & MH532550), a genomically stable and phenotypically safe probiotic bacterium shall be used in this clinical trial. The frequency of diarrhea, severity of related symptoms and stool consistency in the intervened group and control group shall be assessed as outcome variables.

Study goals and objectives

To assessed the efficacy and safety of *B. clausii* 088AE in the treatment of antibiotic-associated diarrhoea with related gastrointestinal disorders.

Study design

Study is planned as a prospective, interventional, randomized, double blind, parallel, placebo-controlled clinical study to evaluate the efficacy and safety of *Bacillus clausii* 088AE in the treatment of antibiotic-associated diarrhoea

Study is to be conducted with 120 patients comprising pediatric (60, 2-10 years) and adolescent and adult (60, 11-up to 65 years) patients. Each group shall have two arms, e.g., placebo and test arms (30 patients in each arm).

1. Placebo pediatric AAD (n=30 randomized)
2. Test pediatric AAD (n=30 randomized)
3. Placebo adolescent & Adult AAD (n=30 randomized)
4. Test adolescent & Adult AAD (n=30 randomized)

The total duration of the study for patients shall be 17 days. 60 Patients shall receive placebo and 60 patients shall receive test product as per randomization. All patients shall take either test product or placebo for seven days.

Selection of study population

Inclusion criteria

Subjects who meet the following criteria shall be considered eligible to participate in the study:

- Male and females aged >2 and <65 years completed years (both inclusive) with diarrhoea and to be treated for 7 days.
- Patients shall be treated on physicians prescribed broad spectrum antibiotics, belonging to beta-lactam, lincomycin, cephalosporin and macrolide class
- Willing to give written informed consent/assent form by study participants or parent wherever applicable.

Exclusion criteria

The subjects who has following criteria shall be excluded from the study

- Patients with bloody or purulent stool, with pus or mucus.
- Severe dehydration needing hospitalization.
- An axillary temperature greater than (>) 38.2°C or an oral temperature > 38.6°C
- Symptoms of septicaemia (fever, shivering, or feeling cold, fast heart rate, fast breathing and shortness of breath, sweaty or clammy skin, etc.)
- Unable to take medication orally or tolerate oral rehydration.
- Taken probiotics prior to study (2 weeks) or during study other than interventional product.
- History of gastric ulcer, duodenal ulcer, combined gastric and duodenal ulcers, upper gastrointestinal bleeding, autoimmune gastritis and GERD.
- Use of any proton pump inhibitor, sucralfate, H_2-receptor antagonist, or bismuth preparations within 1 week before initiating study IP therapy.
- Use of any muscarine receptor antagonist and gastrin receptor antagonist within 2 weeks before initiating study IP therapy.
- History of intubations for co-morbidities (chronic lung disease (CLD), congenital heart defects (CHD) or neurologic disorders.
- Use of any other investigational drug currently or within 30 days prior to study entry

Withdrawal criteria

The patient shall be withdrawn from the study by the investigator for any of the following:

- Occurrence of an adverse event, associated with the administration of the IP and requiring its cancellation.
- Emergence of any diseases or conditions during the study that worsen the prognosis of the patient, as well as make it impossible for the patient to continue his/her participation in the clinical study.
- The need for a forbidden concomitant therapy.
- Pregnancy of the patient.
- Violation of the study protocol, like - improper inclusion of the patient who did not meet the inclusion criteria and/or met the relevant exclusion criteria; other violations of the protocol, which, according to the investigators, are significant and withdrawal of the informed consent by the patient.

Methodology

Method of assigning patients to treatment groups
Upon enrolment in the study, patients shall be randomly assigned to either placebo or test products. 30 Patients in each treatment arm shall be assigned. The treatment assignment shall be based on randomization sheet generated using SAS version 9.1.3 by the random number generation method. Investigator shall remain blind till the completion of the study. If there is any adverse event or other emergency requiring knowledge of subject condition assignment, the blinding can be broken for individual subject using the envelope method. Un-blinding shall be done only at the end of clinical phase completion.

IP, forms and treatment dose
The investigational product is spore preparation (powder) of *B. clausii* 088AE with activity 2 billion spores per gram with maltodextrin as excipient. Both investigational product and placebo product are in sachet form (1.00 g/sachet). Both the product complied the activity standard and standard microbial limit test following internal quality control and quality assurance measures. Physical appearance and labelling are identical. Pediatric patients shall receive 2 sachets per day (IP or placebo) and adolescent and adult patients shall receive 3 sachets per day (IP or placebo).

Randomization, blinding and unblinding
A randomization list with drug codes is followed for patients enrolment at the site. A randomization list shall be generated for this study by using the statistical program in the SAS environment by the random number generation method. Patients allocation shall follow the randomization numbers allocation order of assigning in an increasing order (for example, after the patient with the number "101", the next patient would be assign the number "102" etc.). After reviewing the randomization sheet, the physician-investigator shall define therapy for the patient. The randomization sheet shall contain information about the sponsor of the study, protocol number, randomization number and the information concerning the assignment of code of (B) *Bacillus clausii* 088AE or (A) placebo to the patients corresponding to his/her randomization number. The investigator and patients shall not be aware about the treatment group and placebo group.

The blinding will be broken post data compilation. The details about the investigational product and comparator product will be informed to the principal investigator in the case of SAE. The blinding will be broken by the sponsor and /or site PI the particular subject blinding will be revealed to the principal investigator and other responsible persons to provide the further medical treatment.

Concomitant medications
Antibiotics classes which induced the diarrhea are given as concomitant medications during the study period other than treatment medications. Following class of antibiotics are used, e.g., beta-lactam, lincomycin, cephalosporin and macrolides.

Treatment compliance
Patients are asked to follow the instructions of the investigator and/or the person entitled by the investigator. The administration of treatment products are recorded in a diary card and checked by the investigator and/or the person entitled by the investigator in each visits.

Study plan
The safety and efficacy evaluation of *B. clausii* 088AE in antibiotic-associated diarrhea (AAD) followed the following schedule of events -

Presentation of information and consent document → Visit-1 (screening, randomization

and initiation of treatment, 1st day) → Visit-2 (continuation of treatment, 2nd day) → Visit-3 (continuation of treatment, 3rd day) → Visit-4 (continuation of treatment, 5th day) → Visit-5 [end of treatment (EOT), after 7 days of treatment, 8th day) → Telephonic follow up (17±3rd day). A detailed schedule of events are as follows (Table 1) –

Table 1. Schematic schedule of the clinical trial to evaluate the safety and efficacy of *Bacillus clausii* 088AE on antibiotic-associated diarrhea (AAD) patients.

Visits	Visit 01 (Day 1)[a]	Visit 02 (Day 2)[b]	Visit 03 (Day 3)[c]	Visit 04 (Day 5)[d]	Visit 05 (Day 8)[e]	Visit 06 (Day 17±3)[f]	Visit 07[g]
Informed consent/assent	√						
Demography	√						
Medical/surgical history	√						
Inclusion/exclusion criteria	√						
IP Dispensing	√	√	√	√			
Issue diary card	√	√	√	√			
Physical examination	√	√	√	√	√		
Vital signs	√	√	√	√	√		√
Investigator assessments for severity of diarrhea	√	√	√	√	√		
Laboratory tests	√				√		
Compliance check		√	√	√	√		
Primary endpoints		√	√	√	√		
Secondary endpoints		√	√	√	√		
Concomitant medications		√	√	√	√	√	√
AE/SAE assessment		√	√	√	√	√	√

[a] Visit 01 shall be for screening, randomization and initiation of treatment (baseline); [b] Visit 02 shall be second day of treatment; [c] Visit 03 shall be third day of treatment; [d] Visit 04 shall be fifth day of treatment; [e] Visit 05 shall be eighth day of treatment, i.e., end of treatment (EOT); [f] Visit 06 shall be on 17±3 day and for telephonic follow up and [g] Visit 07 shall be unscheduled visit.

Sample power and appropriateness of measurements

The study is planned on 120 patients, i.e., with an ITT (intension to treat) population of 120 patients. Each group with sixty subjects presented 90% sample power to detect non-inferiority (NIM, -0.170; α=0.050 when MD=1.00). Efficacy analyses shall be performed between test and placebo arms within the group for primary and secondary end-points, separately. This shall be done in order to evaluate the efficacy of the *B. clausii* 088AE compared to placebo in the respective groups.

Primary efficacy variables(s)

Efficacy analysis shall be performed for the per-protocol (PP) population and primary efficacy analysis is based on PP patient's samples. Analysis of the PP population is essential to prove the study hypothesis.

Primary Endpoints

- Time to Last Unformed Stool (TTLUS) – At 24 hr, 48 hr, 96 hr, 168 hr.
- Number of unformed stools at time points - At 24 hr, 48 hr, 96 hr, 168 hr – starting from first IP administration.
- Time to complete resolution of functional gastrointestinal discomforts- At 24 hr, 48 hr, 96 hr, 168 hr
- % responders as defined by the number of subjects with complete remission of diarrhea within At 24 hr, 48 hr, 96 hr, 168 hr

Secondary Endpoints

- To evaluate the changes of the severity of AAD related symptoms [Time Frame: Baseline/Day 1 to EOT]
- To evaluate the changes in VAS score of AAD At 24 hr, 48 hr, 96 hr, 168 hr
- Tolerance [Time Frame: Baseline/Day 1 to EOT]
- Assessment of safety of the Investigational products
    - Adverse event(s)
    - Physical examination
    - Biomarker of systemic safety
        Hematological test (complete and differential blood count)
        Liver function test (SGOT, SGPT)
        Renal function test [serum creatinine, serum albumin, serum sodium, serum potassium, blood urea nitrogen (BUN)]
        Biochemical test (RBS, total cholesterol)

Endpoints Analysis: Efficacy and Safety Variables

Primary and secondary endpoints shall be evaluated on different efficacy and safety variables of *Bacillus clausii* 088AE. Different endpoint results of test arm shall be compared with the respective placebo arm within specific population group, i.e., paediatrics or adolescent and adults. In primary endpoints, the efficacy of *Bacillus clausii* 088AE shall be evaluated for time to last unformed stool (TTLUS), total number of unformed stools, time to complete resolution of functional gastrointestinal discomforts and percentage (%) of responders with complete remission of diarrhea at different treatment durations (24 - 168 h).

Secondary endpoints shall be evaluated on changes of severity of AAD related symptoms, visual analog scale (VAS) and tolerance to investigational product at baseline to EOT. Severity of AAD shall be assessed by physician's investigation (scores) for bloating, distension, flatulence, odorous flatulence, difficult gas evacuation, stomach rumbling, belching, bad breath, abdominal movement and excessive gas evacuation. The mean severity score shall be calculated from a four-point scale on symptoms (0=none, 1=mild, 2=moderate and 3= Severe). AAD-associated symptoms like abdominal pain, bloating and flatulence, vomiting and nausea, perception of mental wellbeing and influence on daily life shall be assessed through visual analogue scale (VAS, 0-10). Safety of *Bacillus clausii* 088AE shall be assessed by examining general physical health, systemic biomarkers and adverse effect reports. The clinical safety shall be assessed through systemic biomarkers like hematological (complete blood count), hepatic [aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT)], renal function test (serum concentration of creatinine, albumin, sodium, potassium and blood urea nitrogen), random blood sugar (RBS) and total cholesterol at baseline and EOT. Biomarkers analysis shall be performed following the standard medical test protocols (ICMR guideline). The adverse effect (AE) is defined as any medically untoward event detected in a clinical study subject after use of the study agents, whether or not caused by the use of the agents. Whereas, serious adverse event (SAE) is defined as any untoward medical incidence which is life-threatening and results into death or hospitalization, disability or incapacity and congenital anomaly.

Collection of blood samples, analytical methods and data collection

Blood samples to be collected from enrolled patients on first visits (before treatment begin at baseline) and fifth visit (day 8, end of treatment) following standard phlebotomy practices following WHO guidelines. Clinical assessment shall be carried out in certified clinical and pathological laboratory. Standard clinical analytical procedures to be followed for clinical samples assessment. Standard laboratory assessment reports to be checked by registered

pathologist/clinician authorized by principal investigator. Original test reports shall be submitted to the sponsor along with clinical study reports.

Planned number of subjects and sample size justification

This study is to evaluate the safety and efficacy IP from baseline to EOT of to prove that on Day 20 the mean clinical change between baseline and EOT will be statistically significant. The necessary number shall be calculated, basing on 90% power and 5% significance (unilateral). A total of sixty patients are required to have a 90% chance of detecting, as significant at the 5% level.

The calculation is done based on the following formula:

$n = f(\alpha/2, \beta) \times [p_1 \times (100 - p_1) + p_2 \times (100 - p_2)] / (p_2 - p_1)^2$

where p_1 and p_2 are the percent 'success' in the control and experimental group respectively, and

$f(\alpha, \beta) = [\Phi^{-1}(\alpha) + \Phi^{-1}(\beta)]^2$

Φ^{-1} is the cumulative distribution function of a standardized normal deviate

Safety considerations

Definition, severity and relationship with the study product, types of adverse events

Adverse event (AE) is any medically untoward event detected in a patient or clinical study subject after use of a medicinal product, whether or not caused by use of the product. An adverse event can be any untoward symptom (including an abnormal laboratory finding), complaint, or disease that may be temporally associated with use of a medicinal (study) product, whether or not related to use of the product. During the study all the new events, adverse events that occurring to the patient while using the IP will be documented. Adverse events include changes in laboratory findings in case the latter are clinically significant and require medical intervention. AE severity is determined as:

Mild	If signs or symptoms do not affect daily routines and do not require medical intervention;
Moderate	If it is intense enough to affect daily routines and medical intervention may be required;
Severe	If a study subject fails to work or conduct his/her daily routines and medical intervention is necessary.

Relationship between an AE and the study IP is determined as:

Definite	if AE clinical manifestation(s) and/or laboratory abnormalities occur after use of the IP, and cannot be explained with existing conditions and other factors and if an AE is improved on withdrawal of the IP and reappeared on re-exposure;
Possible	If AE clinical manifestation(s) and/or laboratory abnormalities are temporally related to use of the IP, and are unlikely to be associated with existing conditions and other factors and if an AE is improved on withdrawal of the IP and reaction to re-exposure is unknown;
Probable	If AE clinical manifestation(s) and/or laboratory abnormalities are temporally related to use of the IP but can be explained with existing conditions or exposure to other IPs and chemical substances and if reaction to withdrawal of the IP is unclear;
Unlikely	If AE clinical manifestation(s) and/or laboratory abnormalities are not definitely temporally related to use of the IP and if there are other factor (IPs, conditions, chemical substances) that can cause them;
Unclassified	If AE clinical manifestation(s) and/or laboratory abnormalities are difficult to assess, and if additional data are necessary or those data are under examination;
Unclassifiable	If reports report suggesting AEs cannot be judged because of insufficient or contradictory information.

Types of adverse events:
Serious adverse event (SAE) is any unfavorable medical event that, at any dose, like results in death, or is life-threatening, or requires hospitalization or prolongation of existing hospitalization, or results in pertinent or insignificant disability or incapacity, or is congenital anomaly or birth defect.

Adverse reaction (AE) (as related to a pre-marketing clinical study of use of new medicinal product or its use for new indications, especially in case its therapeutic range has not been specified yet) is any untoward reaction associated with any dose of the product.

Unpredicted adverse reaction is an adverse event which or severity of which is not consistent with known product information.

Registration of adverse events
Adverse events will be documented on CRF special pages. For any AE information documented in the source document (medical history, case history) should include:
- Symptoms;
- Severity (mild, moderate, severe);
- Type of relationship with exposure to the study IP;
- Term (initiation and termination dates).

In case of a serious adverse event, the investigator must:
1. Inform the study monitor on SAE and send the filled SAE form via fax/email within 24 h after being informed; then the monitor must inform the person in charge of pharmacovigilance authorized by the Sponsor. The study Sponsor will inform regulatory bodies on SAE. Information included in the initial report on occurrence of a serious adverse event should contain at least report on occurrence of a serious adverse event, patient's identifying data as related to the clinical study, identifying data of the person that reports on occurrence of the event (i.e. the investigator or sub-investigator), characteristic of an event occurred and its causal relationship with use of the study IP. Any SAE occurred from initiation of IP use till end of participation in the study should be documented and reported.
2. Inform the patient on withdrawal of the IP and monitor him/her until improvement or stabilization of his/her state.

The PI will report all SAEs to the DCGI, sponsor and the Ethics committee (that approved the protocol) within 24 hours of their occurrence. The report of any serious adverse event of permanent injury or death after due analysis shall be forwarded by the PI to chairman of ethics committee, chairman of expert committee constituted by Drugs controller general of India, with a copy of report to Drugs controller general of India and Head of the trial site within fourteen days of occurrence of serious adverse event of permanent injury or death.

The report of any serious adverse event other than death after due analysis shall be forwarded by the sponsor to Drugs controller general of India, Chairman of Ethics committee and the Head of the trial site within fourteen days of occurrence of serious adverse event.

For the recording of signs and symptoms, the patient will be required to report spontaneously any AEs as well as the time of onset and the intensity of these events. Signs and symptoms will be recorded as AEs if reported spontaneously by the patient. In addition, each patient will be asked by the investigator for AEs during vital & physical examination. All adverse events, independent of relationship to study IP, must be reported starting from time of first administration of study IP until 30 days after the end of treatment visit. All abnormal values occurring before dosing but after signing the ICF will be considered as medical event

and recorded in medical history and current medical condition, as applicable. Each Adverse Event will have following information:

- Protocol number
- Patient identification number
- Description of the symptom / event
- Classification “serious” or “not serious”
- Intensity
- Start date and stop date
- Treatment/medication required.
- Causal relationship
- Outcome of event (recovered without sequelae [disability/incapacity], recovered with sequelae, and not yet recovered death [with date and cause recorded] and unknown).
- The action(s) taken

Criteria for reporting

Adverse Events will be recorded from the time of first administration of study IP until 30 days after the end of treatment visit. Events occurring before starting study treatment but after signing the informed consent form are recorded as Medical Event on the Medical History/Current Medical Conditions of Case Report Form. The SAEs must be reported to the sponsors immediately (within 24 hours of the awareness of the event). The investigator must complete, sign and date the SAE form, verify the accuracy of the information recorded on the SAE form with the corresponding source documents, and send a copy (by fax/mail) to sponsor/representative. All SAE analysis report will be reported within 10 calendar days to the regulatory agencies (DCGI) by the sponsor.

Follow-up of Adverse Events

All AEs will be followed to resolution (the patient's health has returned to his/her baseline status or all variables have returned to normal), an outcome is reached or till stabilization (the investigator does not expect any further improvement or worsening of the event) of the event. Wherever appropriate, medical tests and examinations will be performed to document resolution of event(s).

Management /follow up of SAEs

The investigator should institute any supplemental investigations of SAE based on their clinical judgment of likely causative factors. If required, a follow-up report including all relevant new or reassessed information (e.g., concomitant treatment, medical history) obtained on the SAE / will be prepared and same will be marked “Follow-up report”. This report will be sent to the sponsor. Recorded for any trial participant after study IP administration should be reported immediately to the sponsor/representative. Every effort will be made to gather information regarding the outcome until 8 weeks post-partum/termination of. It will be the responsibility of the investigator to obtain this information.” recorded after signing the ICF (before IP administration) will be reported. However, form need not be filled for the same. Also, the patient will be withdrawn from the study and no further follow up will be done in such cases. In case of after IP administration, separate forms (Form A and Form B) should be filled. If a patient dies during participation in the study and an autopsy is performed, a copy of the report must be submitted.

Data management and statistical analysis

To provide independent evaluation of the results obtained, statistical data analyses will be performed by Biostatistician. A primary database will be created in validated Microsoft Excel spreadsheets while processing registration forms received from the study sites. Statistical analyses of the data will be performed using statistical software SAS® version 9.1.3 all deviations from the final version of the statistical analysis plan will be described and substantiated in the final report. Any posterior or interim analysis, if performed will be substantiated and documented in the statistical report.

Descriptive statistics will be presented for all continuous efficacy and safety indicators obtained during the study and frequency distribution will be presented for all categorical variables available in the data. Normality of the data will be tested with one of standard methods (Shapiro-Wilk test or Kolmogorov-Smirnov test). Parametric tests will be used for the variables following normal distribution. In case the data set variables found of non-normal in nature nonparametric methods will be used to compare efficacy and safety indicators (31).

Analysis of demographic and disease data, concomitant therapy and other baseline data
Demographic data (age, sex) and baseline data will be presented with frequency distribution and descriptive statistics, depending on the variable type (continuous, categorical). Following descriptive statistics will be reported for continuous variables like mean, standard deviation (SD), median, interquartile range, minimum and maximum.

To test hypothesis of homogeneity of groups at baseline, absence of differences between groups with Student's t-test and ANOVA (for interval parameters with normal distribution in the population under consideration), Wilcoxon-signed rank test (for ordinal interval parameters with non-normal distribution) or χ^2 test (for attributes) will be used. In case statistically significant differences are found, difference between treatment groups will be estimated with use of 95% confidence intervals at 5% level of significance. ANCOVA may also be used to compare the scores, if required. Separate analyses will be performed for Primary and Secondary end-points.

Efficacy analysis
Efficacy analysis will be performed for the per-protocol (PP) population. Primary efficacy analysis will be based on PP patient's samples. Analysis of primary and secondary endpoints the PP population will be essential to prove the study hypothesis. To test null hypothesis of presence of differences between the groups in the primary efficacy endpoint, Student's t-test is to be used for independent samples following normal distribution. In case data is found to follow non-normal distribution, the non-parametric Wilcoxon Signed-rank test will be used. All the efficacy endpoints will be also compared between the study groups. Comparison of groups in the endpoint on treatment completion will be conducted with Student's t-test or Wilcoxon Signed-rank test (for indiscrete parameters, depending on accepted conclusion of attribute distribution). All the secondary efficacy endpoints will be also compared between the study groups. Comparison of groups in the secondary efficacy endpoints will be performed with χ^2test.

Safety analysis
Safety analysis will be performed, based on following safety endpoints like prevalence of AEs stratified by severity and frequency, incidence of AEs associated with use of the study IP, incidence of SAEs associated with use of the study drug, incidence of patients with at least one documented AE, proportion of patients stopped treatment due to AEs.

AE/SAE prevalence and amount of patients with AEs/SAEs (percentage of safety analysis population) will be calculated. AE severity and relationship with the study treatment (according to the investigator). Information on incidence of AEs/SAEs associated by

the investigator with use of the IPs. All the safety data will be compared between arms within the study groups. To compare AE/SAE incidence in the treatment groups, χ^2test is to be applied. To compare AE/SAE severity and possible casualty between AEs/SAEs, Mann–Whitney U test is to be applied.

Interim analysis
Interim analysis will not be performed.

Applied significance level
Significance levels and confidential intervals are to be calculated as bilateral, and difference statistical significance will be bilateral by default and relate to significance level of 0.05 (unless otherwise specified).

Procedures for accounting missing and dubious data, and data that are not subject to analysis
In case of missing and dubious data, and data that are not subject to analysis, appropriate methods of statistical analysis will be applied and missing data for the subjects will not analyzed and not replaced by any other data. Procedures for reporting of any deviations from the original statistical plan. All deviations will be described and justified in the final study report.

Selection of patients for statistical analysis (populations subject to analysis)
Statistical analysis will include the following populations:
Safety population: patients that received the IP, for whom there is assessment at least for one post-dose time point for AE evaluation.
Per protocol (PP population): patients completed the study per study protocol. Analysis will be performed for PP population analysis will be essential to decide whether the study hypothesis has been proved.

Quality assurance

Site qualification
The investigator should have education, professional training and experience to take responsibility for proper conduct clinical study. The investigator should know in detail how to use study IPs in accordance with the protocol, the current brochure revision, information on the IPs and other sources provided by the Sponsor. The investigator should keep a journal/list of duties distribution, i.e. a list of properly qualified persons who will conduct certain activities during the study by their order.

Study monitoring
Clinical study monitoring will be performed by the Sponsor. The monitor of the clinical study will be responsible for compliance with the requirements of Good Clinical Practice and the national regulations. The investigator should provide the coordinator with an opportunity to visit the study site for evaluation of its compliance with the protocol requirements. Before study initiation the coordinator should bring an initial visit to be sure that all the materials (CRFs, study IPs, etc.) will be received at site in proper condition and that the investigator and their staff shall be instructed on procedures and protocol requirements.

During the study

Study site monitoring will be conducted by visits, telephone calls and CRFs check, frequent enough to evaluate rate of inclusion, CRFs data completeness and accuracy, and AEs occurrence. The clinical study monitor will bring monitoring visits in accordance with the schedule prepared beforehand, frequently enough to verify CRFs data in comparison with source data and to control compliance and handling with the study IP. Upon completion of all subjects participation the monitor will bring a final visit to solve problems remained and agree on sending the rest of study materials (CRFs, study IPs) to the Sponsor.

The investigator should provide the coordinator and other Sponsor's representatives with opportunities to:

- Visit the study site and have access to the rooms where the study is conducted and to the study materials;
- Meet study staff and have access to the study documentation;
- Check whether CRFs are correctly fulfilled and compare them with source documents;
- Control compliance with the protocol in terms of course of the study.

All the information related to study monitoring will be considered as strictly confidential.

Inspection and audit

National regulatory bodies may conduct inspections during and after the study. Besides, Sponsor may conduct audits of this clinical study. Audit can include (not to be limited to) checking correctness of storage and dispensing of the study IP, presence of necessary documents, compliance with PIL+ICF signing procedure, keeping medical documents, compliance with the study protocol in general and comparison of CRFs data with source documents.

Dissemination of results and publication policy

The sponsor has exclusive right for study outcomes and sole publication.

Project management

Name	Role
Dr. Chiranjit Maity	Trial management
Dr. Anil K. Gupta	Trial management
Dr. Harish S.	Trial coordination
Dr. Giri Raja K V	Clinical investigation
Dr. Prashant Sadashiv Deshmukh	Clinical investigation

Ethics

The study will be conducted according to the current version of the Declaration of Helsinki (Brazil 2013) and in compliance to the current ICMR Guidelines for Biomedical Research on Human Patients, New drugs & clinical trials rules 2019of IP and Cosmetics Act, ICH-GCP Guidelines (1996 & 2002) and other applicable regulatory guidelines.

Ethics committee and local ethics committee

The Sponsor will provide a copy of the Protocol and other necessary study documentation to the Ethics Committee of the country where the study is being conducted. The study cannot be initiated before the investigator has got approval from the Ethics Committee and other applicable formal requirements have met.

Patient information sheet/ Subject Information Sheet
The investigator will inform any potential study subject on the study IPs, study purpose and essence, expected benefits, degree of risks and requirements a patient should meet during the study. Each patient will be handed information on the study in printing, outlined in plain language. No patient will be included in the clinical study until he/she is properly informed and has enough time to think over whether he/she will participate in this clinical study. Consent should be written before a patient is exposed to any procedures related to the study. Patients will be included in the study if they have written the informed consent form of the patient information leaflet. The informed consent form of the patient information leaflet will be signed by both a patient and the investigator in two copies. One copy will be handed to the patient; the other will be kept at the study site.

Protection of personal data of the patient
The investigator should fulfill and keep the patients screening journal and the identifying list of all the included patients. Handling with information will comply with professional secrecy requirements. The investigator and the Sponsor will provide protection of personal data of the study subjects. Necessary personal data of subjects, such as social and demographic parameters, will be collected minimally and exclusively for study purposes. No documents identifying patients will be publicized. First names and surnames of patients will not be reported to the Sponsor. Before inclusion in the study, patients will be informed on confidentiality conditions and use of their personal data, particularly on access to them by the monitor and other staff in charge (in case of audit, inspection, etc.). These conditions will be outlined in the information for patients.

Data collection
Any information on patients obtained during this study will be first documented in the source documents and then in the case report form (CRF) the investigator has. These forms have been developed to document clinical and laboratory findings as per Protocol, with space for additional comments. CRF originals are ownership of the Sponsor and available for their representatives only. Upon study completion CRF originals will be transferred to the Sponsor's representative. The investigator should retain a copy of the CRFs for their archive. All the CRF sections should be filled completely. If data on some issue is missing, this should be documented in the Section 'Visit Comments'. Mistakes should be crossed, so that they might have been read. No blur-over is allowed. Correct information should be written above, below or besides, depending on spare space. Each correction should be dated and signed by the person making the entry. The investigator should provide safe-keeping and integrity of records and documents concerning conducting the study, use of the study IP and other important information including CRFs, signed PILs with ICFs and information on IP accountability The Sponsor should inform the investigator on any changes in the study scheme trough their representatives.

Storage of documents
The investigator will store/archive all the study documents, including original patients' medical documents, for at least two years after the study IP registration (upon Sponsor's notice) or longer if that is required by applicable regulations. The documents concerning the study will be available for regulatory bodies' inspection/Sponsor's audit.

Disposal of study documents is possible with Sponsor's written consent. The Sponsor will inform the investigator of expiration of term of documentation storage in writing.

Primary documents, which enable to evaluate conducting the study and quality of the data obtained, will be stored to provide their safe-keeping and integrity throughout the storage term as well as their accessibility at request of the authorized bodies.

Insurance

Safety and well-being of study subjects will be ensured. Before study initiation, an insurance company shall make an agreement of obligatory life and health insurance for study subjects with cordinator. The study subjects will be covered under a clinical trial insurance policy from insurance Company for treatment of adverse events and payment of compensation in the event of clinical trial injury or death. In the event of permanent injury/death, the patient will be compensated by the insurance company as per current provisions of new drugs & clinical trials rules 2019.

Informed consent forms

Written and oral information shall be provided to all patients in both English and translated native language. Every subject shall give written informed consent to investigator after understating the objective of this trial, including possible risks and benefits.

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PART 2

Budget

The efficacy and safety of Bacillus clausii 088AE in the treatment of antibiotic-associated diarrhoea
Advanced Enzyme Technologies Ltd., Thane

Not applicable

Other support for the project
Not applicable

Collaboration with other scientists or research institutions
Not applicable

Links to other projects
Not applicable

Curriculum Vitae of investigators
Not applicable

Other research activities of the investigators
Not applicable

Financing and insurance
The study shall be solely funded by Advanced Enzyme Technologies Ltd., and not received any other external funds.